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Gene therapy with recombinant adenovirus vectors: evaluation of the host immune response

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Abstract

E1, E3-deleted, replication-deficient recombinant adenoviruses are widely studied as vectors for their capacity to transfer therapeutic genes in vivo. They can infect a wide variety of dividing and quiescent cells from different organs and possess a large packaging capacity. One of the major limitations in the use of these vectors for gene therapy is the transient expression of the transgene in vivo and the poor transduction efficiency when re-administered. Despite the deletion of the viral E1 region, low level of early and late viral genes are expressed in vivo. Thus, viral antigens plus those derived from transgene expression in transduced cells contribute to cellular immune responses leading to the destruction of these cells. Production of anti-adenovirus antibodies, the cellular immune response as well as the early non-specific clearance of the vectors, constitute barriers to successful gene therapy. New vectors have been derived with additional deletions in the E2a or the E4 regions. Such second generation vectors were evaluated in vivo. These studies have revealed the complexity of the immune mechanisms elicited by these vectors and the importance of several parameters in these evaluations (i.e. mouse strains, nature of the transgene, route of administration...). In order to inhibit the production of neutralizing antibodies to adenovirus that prevent from further readministration of the vectors, immunosuppressive strategies were undertaken. Treatment regimens with immunosuppressive drugs (cyclophosphamide, FK506) or with monoclonal antibodies that block either the T cell receptor or costimulation pathways allow prolonged transgene expression and/or readministration of adenoviral vectors. In addition, transduction efficiencies may be increased by transiently inhibiting non-specific immune mechanisms that lead to the dramatic early clearance of the vectors. Taken together, these strategies may improve further gene therapy protocols by decreasing the host immune response to adenoviral vectors. © 1997 Elsevier Science B.V.

Keywords: Gene therapy; Adenovirus; Immune response

1. Introduction

Human adenoviruses (Ad) have been the focus of considerable attention as gene transfer vectors due to their ability to efficiently infect a wide variety of cell types both in vitro and in vivo. Most current recombinant vectors are generated by inserting the therapeutic gene in place of the essential E1 genes and thus must be propagated in a complementation cell line providing in trans the missing E1 proteins. Such vectors are therefore replication-deficient in the host but can be produced at high titers using appropriate complementing cell lines [1,2]. The ability of E1-deleted vectors to efficiently transduce in vivo both dividing and quiescent cells from various organs, such as liver [3], lung [4], muscles [5–7], and to express the inserted transgene at high levels has been considered as one of the major advantages of the adenovirus-derived vectors over other gene transfer vectors.

Various recombinant adenovirus vectors have been produced for the development and evaluation of potential genetic treatments of inherited or acquired diseases. For instance, the natural tropism of the adenovirus for the lung was exploited for in vivo transfer of the human cystic fibrosis transmembrane conductance regulator (CFTR) gene into the airway epithelia of humans and

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nonhuman primates [8,9]. Initial encouraging gene transfer efficiencies and limited side effects observed in certain animal models led to the initiation of several clinical trials in cystic fibrosis patients [9]. Likewise, studies have also been undertaken for the treatment of neuromuscular diseases such as Duchenne Muscular Dystrophy, using adenovirus vectors expressing the dystrophin gene [10]. Injection of these vectors into mice lacking dystophin (mdx mice) induced a correction of the defective phenotype [6,11]. Other genetic diseases such as hemophilia or hypercholesterolemia have also been considered as potential targets for adenovirus-mediated in vivo gene therapy [12,13].

However, in vivo experimental evaluation of recombinant E1-deleted vectors have revealed several limitations which might seriously impair the use of such recombinant vectors in gene therapy protocols.

1.1. Specific cellular immune responses to recombinant adenoviral vectors and transgene's product

Over the last 2 years, numerous reports have shown that in vivo expression of the transgene was in most cases only transient [14,15]. Intravenous injection of E1-deleted vectors into immunocompetent mice allows a transgene expression in the liver that usually reaches a peak level at 2–3 days post-administration but declines rapidly to almost undetectable levels after 3–4 weeks. In contrast, a relatively stable expression of the transgene is observed in immunodeficient strains treated with the same procedure.

While initial studies demonstrated that short-lived in vivo expression of the transgene was most probably related to the induction of a host immune response directed against the adenovirus antigens [15–19], the role of the transgene derived product itself was not investigated until recently. Most of the in vivo studies were done with recombinant E1-deleted vectors expressing bacterial or human proteins that can act as potent antigens and contribute to the cellular immune response leading to the destruction of the transduced cells. Indeed, injection of an adenoviral vector expressing the LacZ gene was shown to induce a potent cytotoxic T lymphocyte response (CTL) directed against the β-galactosidase antigen [20]. Similarly, Tripathy et al., 1996, demonstrated in the mouse a very transient expression of the human erythropoeitin. In contrast, injection of a recombinant adenovirus vector encoding the 'self' non-immunogenic murine erythropoietin allows a stable in vivo expression of the transgene [21].

In order to more precisely elucidate the respective roles of the anti-transgene versus the anti-virus immune response in the transient persistence of transgene expression, we investigated in nine different immunocompetent and immunodeficient strains of mice the influence of the mouse model, nature of transgene (bacterial β-galactosidase versus human FIX), type of immune response (humoral versus cellular) and targets of immunity (transgene-encoded products versus adenovirus antigens) on the in vivo persistence of the transduced cells. We demonstrated that clearance of the adenoviral DNA in the liver and lung was more pronounced with a vector carrying the LacZ transgene (AdLacZ) than with a vector carrying hFIX coagulation factor (AdhFIX) [14]. In the case of AdLacZ, our data suggest that the CTL directed against the transgene derived product constitutes the major determinant in the elimination of the transduced cells [20]. In contrast, cells transduced by AdhFIX were not eliminated despite the induction of a detectable anti-adenovirus CTL reaction (Fig. 1). However, this relatively stable persistence of the AdhFIX-transduced cells did not result in a stable detection of the human FIX coagulation factor. An analysis of various immunological parameters showed that the observed clearance of plasmatic hFIX was correlated with the induction of antibodies directed against hFIX in all tested immunocompetent mice, except in C57BL/6 animals. Such antibodies most probably constitute immune complexes with hFIX, leading to a rapid clearance of the circulating human coagulation factor. In C57BL/6 mice, in which these antibodies

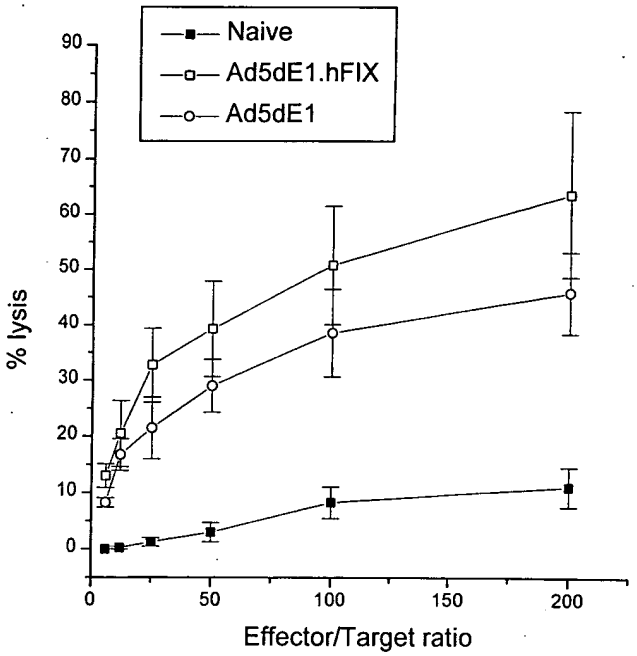

Fig. 1. Anti-adenovirus CTL activity in CBA mice. CBA mice were injected intra-peritoneally with vehicule (naive), 5×10^8 plaque forming-units (pfu) of empty first generation adenovirus (Ad5dE1) and 5×10^8 pfu of first generation adenovirus bearing human factor IX as a transgene (Ad5dE1 hFIX). They were boosted 2 weeks later with the same dose and killed 4 days after the boost. Splenocytes were stimulated in vitro with Ad-infected L929 cells for 6 days. CTL activity was evaluated by a standard 51Cr-release assay using different ratios of effector to target cells (Ad-infected L929 cells). Results are expressed as the mean ± S.E. of four animals per group of mice.

were never detected, the pattern of hFIX expression was similar to those observed in immunodeficient mice. These results extend those obtained from other groups, and indicate that the mouse strain used for such studies does influence the overall persistence of transgene expression, depending on the ability of the host to develop an immune response to the transgene-encoded protein or to viral-encoded antigens. Recently, Fang et al., 1996, demonstrated that the presentation of adenovirus epitopes might similarly be influenced by the nature of the selected mouse strain: adenovirus antigens are recognized through the H2-Kd or Db allele, but weakly through the H2-Kk allele. However, we and others detected some CTLs directed against the adenoviral antigens in H-2k mice. The reason for such discrepancies remains unclear.

While our studies showed a persistence of transgene expression over approximately 3 months using E1/E3-deleted adenovirus vectors, provided that the treated animals are tolerant to the transgene's product, we did observe the induction of both a specific cellular and humoral anti-adenovirus immune response. Thus, several strategies were assessed to circumvent the immunogenicity of recombinant adenoviruses. Since E1/E3-deleted vectors still express at low levels viral genes encoding early or late proteins, the most widely used strategy to improve such vectors was to further delete other essential viral regulatory genes (e.g. E2A, E4) in order to completely abolish any residual expression of adenovirus genes in the infected cells. We have generated such 'second generation' vectors and showed that, as expected, infection of human cells with E1/E3/E4– or E1/E3/E2A-deleted vectors did not lead to any detectable expression of late viral proteins (M. Lusky, unpublished results). The hypothesis that reduced expression of viral antigens should lead to a blunted immune response and thus, result in extended persistence of the transduced cells was first supported by results from Engelhardt et al., 1994. They have generated vectors bearing a temperature-sensitive mutation that inactivates the E2A protein and, hence, the expression of the viral late genes. They could demonstrate a significant improvement of the persistence of β-galactosidase transgene expression in the liver of CBA mice and lung epithelium of cotton rats using E2A-modified AdLacZ vectors. Interestingly, the same mutation introduced in vectors carrying the human α-antitrypsin or canine coagulation factor IX genes did not show any improvement of transgene persistence in either Balb/c mice or hemophilic dogs [23]. This finding highlights the numerous discrepancies observed between many studies, where, according to the animal model used, significant differences were or were not obtained. Adenoviruses bearing further deletions in the E4 region were also tested. Gao et al., 1996, reported that the E4 deletion stabilizes the expression of the transgene when the immune response against the transgene's product (LacZ) was eliminated by using LacZ transgenic mice. However, no improvement of persistence of transgene expression was observed after injection into non-transgenic immunocompetent animals, confirming the predominant influence of the anti-transgene immune response in the elimination of transduced cells.

In order to eliminate all bias introduced by the use of immunogenic transgenes, we have generated and evaluated second generation vectors devoid of any transgene. Such 'empty' vectors deleted simultaneously in E1, E3 and E4 or E1, E3 and E2A regions were produced using E1/E4 and E1/E2 complementing cell lines, respectively (M. Lusky and K. Rittner, unpublished) and were tested in CBA mice for their capacity to induce adenovirus-specific CTL. This study demonstrated that these second generation vectors did elicit detectable CTL responses against the adenovirus antigens (M. Christ, unpublished results). These data are in contradiction with recently published results showing a decreased anti-viral CTL response in β-galactosidase-transgenic mice injected with a virus deleted in the E1 and E4 region and expressing the β-galactosidase gene [24]. We are currently completing this comparative study of the immunogenicity of first and second generation vectors by analyzing the functional properties of the anti-viral CTLs in splenocyte adoptive transfer experiments.

In parallel to the above studies, a long term experiment was initiated to determine in immunocompetent and immunodeficient mice the in vivo persistence of hepatocytes and pulmonary cells transduced by such modified 'empty' vectors. Our results show no significant differences in the persistence of the transduced hepatocytes 2 months after the intra-venous injection of either E1-deleted or E1/E4-deleted vectors, irrespective of the selected strain of mice (M. Lusky et al., manuscript in preparation). Such results suggest that, in our model, the anti-adenovirus cytotoxic cellular immune response plays only a minor role in the control of the in vivo persistence of the transduced cells, and hence of transgene expression.

1.2. Innate immune clearance of adenoviral vectors in vivo

While administration of recombinant adenovirus vectors into mice allows a relatively stable persistence of the transduced cells despite the induction of a specific anti-viral immune response, the role of the innate immune response in the general efficiency of adenovirus-mediated gene transduction was until recently not seriously addressed. However, recent studies from Worgall et al., 1997, reported that 90% of the recombinant viral genome is eliminated from the liver 24 h after intravenous injection of the vector [25]. This immediate

clearance was shown to be independent of the type of expression cassette and dose of vector administered to the animals. Vector clearance was however most probably related to the action of non specific liver macrophages (Kupfer cells) since the transient depletion of these cells partially enhanced both the persistence of the vectors and delayed their clearance [26].

We similarly investigated the role of the innate immune system in the elimination of the recombinant adenovirus particles by injecting both first and second generation empty vectors in the tail vein of immunocompetent mice. A quantitative analysis of the viral DNA present in the liver, spleen and lung tissues was performed by Southern analysis over the first 70 h post-injection (Fig. 2). In agreement with Worgall et al., 1997, we observed in all tested organs a rapid elimination of 90% of the vectors during the first 24 h, irrespective of the nature (E1/E3–, E1/E3/E4– or E1/E3/E2A-deleted) of the injected vector. These results show that the mechanism(s) of the initial and dramatic decline of viral vectors is also independent of adenoviral backbone.

1.3. Neutralizing anti-viral humoral response

Another potential major barrier to a successful clinical application of recombinant adenovirus vectors is the requirement of repeated vector administrations in certain applications. It was shown that intra-venous injection of adenovirus particles induces a strong neutralizing antibody response that prevents any secondary adenovirus-mediated transduction ([15]; our non-published results). Since the antibody response is induced by the injected virions particles, genetic modifications of the adenovirus vector (e.g. further deletions of regulatory genes) is not expected to inhibit the activation of T helper lymphocytes and formation of neutralizing antibodies. Thus, immunosuppressive strategies may be required to allow efficient transduction upon re-administration. Several immunosuppressive drugs are available today, some of them having already been used in humans to prevent the rejection of allogeneic graft. However, prolonged immunosuppression is associated with serious adverse side effects, and transient immunosuppressive protocols would be more appropriate for gene therapy applications. A transient immunosuppression regimen should be possible given the inability of adenovirus vectors to replicate. All virus antigens should therefore be rapidly processed after the vector administration, and no novel antigens are expected to be synthesized in the transduced cells, given the deletion of several essential viral regulatory genes (e.g. E1, E4).

Various immunosuppressive agents were tested in mice for their ability to prevent the onset of the immune cascade leading to antibody formation. Adminis-

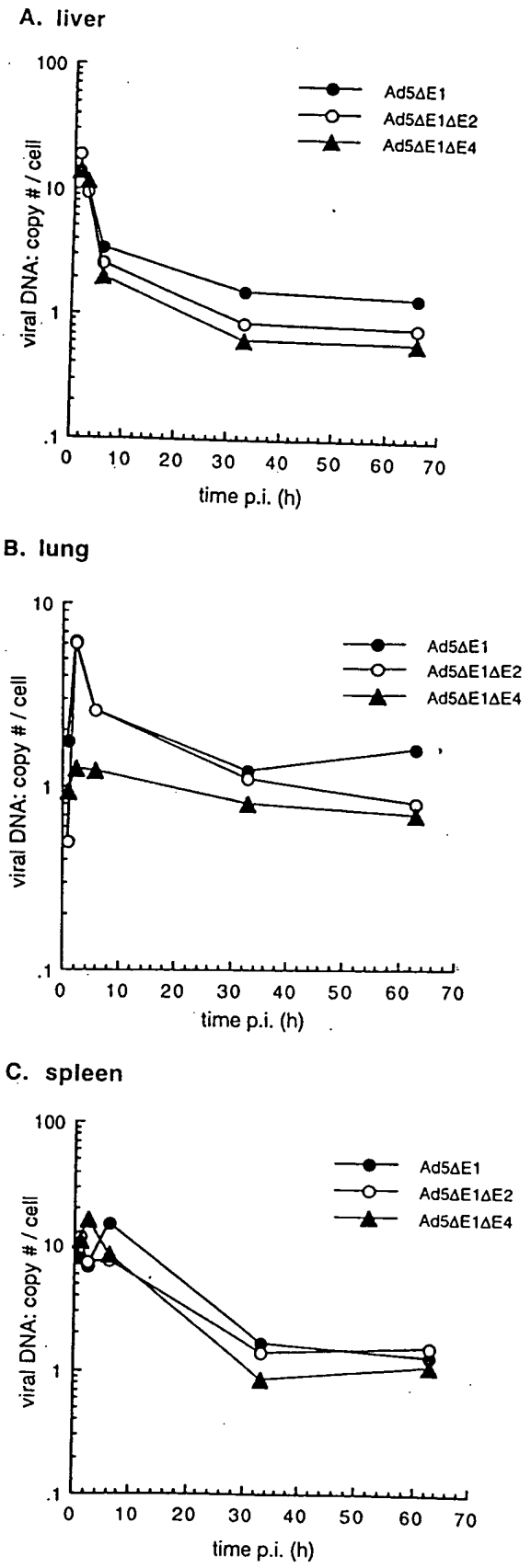

Fig. 2. Non specific immune clearance of adenoviral vectors. Mice were injected (three animals per time point per virus) in the tail vein with 4×10^{10} particles with Ad5dE1, Ad5dE1dE2 and Ad5dE1dE4 viruses. Total cellular genomic DNA was prepared and persistence of the viral DNA was quantified by Southern blot analysis and densitometry scanning of the autoradiographs.

tration of cyclophosphamide around the time of vector injection was shown to stabilize the transgene expression and diminish the mobilization of T cells in the liver and lung of mice injected with adenoviral vectors [27]. This protocol allowed the re-administration of Ad vectors to the lung, but not to the liver. Similar results were obtained with transient FK506 treatment of mice injected in the skeletal muscle with an adenoviral vector expressing the dystrophin minigene with a significant improvement of vector re-administration [28].

In order to identify the most appropriate immunosuppressive agent, we have compared in parallel the effects of a transient treatment of C3H mice with FK506, cyclosporine, dexamethasone or anti-CD4 monoclonal antibodies (GK1.5) on the induction of anti-adenovirus antibodies. We also tested whether a second intra-venous administration into such treated mice of a recombinant adenovirus vector encoding the hFIX protein might lead to an efficient transduction of the murine tissues, and hence, of secretion of plasmatic hFIX, or to a rapid neutralization of the newly injected virions. As shown in Table 1, C3H mice injected i.p. twice with 150 μg of the GK1.5 anti-CD4 antibody one day before and the day of the injection of empty adenoviral vectors, did not develop any anti-adenovirus antibodies. This inhibition is the result of the in vivo depletion of the CD4 population which lasts up to 3 weeks (data not shown, [18,29,30]). Four out of ten mice treated with GK1.5 showed detectable levels of hFIX in the serum after re-administration of AdhFIX (data not shown). In contrast, none of the other transient immunosuppressive regimens allowed a significant decrease of anti-adenovirus antibodies or an efficient re-administration of the adenovirus vector.

Monoclonal antibodies that block either the T cell receptor or costimulation pathways necessary for T lymphocytes activation were similarly used by several groups to lower the immune reactions directed against adenoviral vectors. Anti-T cell receptor antibodies were thus shown to prolong the persistence of transgene expression after adenovirus-mediated gene transfer to mouse synovium [31]. The use of monoclonal antibodies and CTLA4Ig, a ligand of the costimulatory molecule B7, were also shown to successfully prolong transgene expression with first generation adenoviral vectors [32,33]. Studies in mice deficient for CD40L or in mice injected with anti-CD40L pointed out the importance of this molecule and its interaction with CD40 in the induction of a cellular and humoral immune response against adenoviral antigens. Blocking this interaction diminished the production of neutralizing antibodies and allowed re-administration [17,19]. Approaches using such monoclonal antibodies have thus proven their efficiency in various mouse models to blunt both the cellular and humoral arms of the destructive immune response against adenoviral vectors. However, any clinical application of such molecules in combination with adenovirus vectors will require further investigations using more appropriate animal models (e.g. non-human primates).

Interestingly, a recent report showed that administration into rats of an adenoviral vector constitutively expressing the immunomodulatory viral E3 region can lead to a marked decrease of both the humoral and cellular immune responses directed against the virus antigens. E3 proteins have been shown to prevent the development of a strong inflammatory response and to decrease the anti-viral CTL reaction by inhibition of the transport to the cell surface of MHC class I molecules. The precise mechanims responsible for the decrease of the humoral response remain obscure but, as suggested by the authors, the decrease of the inflammatory response and CTL-induced cell lysis might diminish the amount of virus antigens available for a correct presentation and induction of antibodies formation [34]. Whether such modified vectors can represent a valuable alternative to the transient immunosuppressive treatments must still be determined.

Table 1
Anti-adenovirus antibody titers in C3H mice

Groups	Day 23	Day 50	Day 80
Ad5dE1 (A)	8000	4000	800
Ad5dE1dE4 (B)	8000	7000	1750
Ad5dE1+GK1.5 (C)	<100	<250	<250
Ad5dE1dE4+GK1.5 (D)	<100	1250	500

C3H mice were injected intravenously with first (Ad5dE1) and second (Ad5dE1dE4) generation adenoviral vectors. They were treated one day before and the day of adenovirus injection with 150 μg of anti-CD4 antibodies (GK1.5). Serum samples were collected and anti-adenovirus antibodies were measured by ELISA.

2. Conclusion

Most of the ongoing or proposed gene therapy clinical trials using recombinant adenovirus vectors are intended to treat cancer and genetic diseases, in particular cystic fibrosis. While current E1-deleted adenovirus vectors are well suited to gene therapy of cancer, in which a transient expression of cytokines or anti-tumoral cytotoxic proteins is requested, they have been found to be poorly adapted for the treatment of genetic diseases. Despite the deletion of E1, first generation adenovirus vectors were shown to still express late virus genes coding for structural proteins, leading in vivo to the induction of an immune response that rapidly eliminates the transduced cells [15,35]. Moreover, a successful treatment of any genetic disease will probably require a repeated administration of the recombinant vector. Such multiple virus injections will however in-

duce a potent neutralizing antibody response that will significantly decrease the efficiency of transduction.

While the role of the anti-adenovirus neutralizing antibodies in the poor efficiency of a secondary transduction was established without any particular ambiguity, and might possibly be overcome using appropriate transient immunosuppression regimen, the impact of the anti-adenovirus CTL remain controversial.

Our comparative in vivo studies of first (E1-deleted) and second (E1/E2A- and E1/E4-deleted) generation adenovirus vectors have established that: (i) persistence of transgene expression in immunocompetent mice injected with E1-deleted vectors is much better than previously suggested, provided that the animals are tolerant to the protein encoded by the transgene (e.g. hFIX in C57Bl/6 mice); (ii) the type of immune response elicited in the treated animals (humoral versus cellular) significantly influences the persistence of the transduced cells: loss of LacZ expression was found to be correlated with an elimination of the transduced cells, while loss of hFIX detection, in all mice except C57Bl/6 animals, was correlated with a neutralization of the human protein by anti-hFIX antibodies; (iii) although the administration of first and second generation adenoviruses induces a detectable cellular immunity to the viral antigens, the transduced cells persist during a relatively long period of time (over 2 months). The use in previous studies of adenoviruses encoding transgenes of various non-murine origins might have led to an overestimation of the role of anti-adenovirus CTLs in the transient expression of the transgenes in mice. The production of second generation adenovirus vectors by the simultaneous deletion of several regulatory virus genes (e.g. E1 and E4) did therefore not significantly improve the already relatively stable persistence of cells transduced by E1-deleted vectors. Such results are however in contradiction with recent data from Gao et al., 1996, showing an improved in vivo persistence of liver cells transduced with E1/E4-deleted vectors. The reason for these discrepancies remains unclear.

Taken together, these observations highlight the complexity of the immune mechanisms elicited by the in vivo administration of recombinant adenovirus vectors. More studies are required in order to unambiguously determine the respective in vivo properties of E1–, E1/E4 and E1/E2A-deleted adenovirus vectors.

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